Immunohistochemical Assessment of HRAS Q61R Mutations in Breast Adenomyoepitheliomas

Pareja, Fresia ; Toss, Michael S ; Geyer, Felipe C ; da Silva, Edaise M ; Vahdatinia, Mahsa ; Sebastiao, Ana Paula Martins ; Selenica, Pier ; Szatrowski, Austin ; Edelweiss, Marcia ; Wen, Hannah Y ; Mihai, Raluca ; Varga, Zsuzsanna ; Foschini, Maria P ; Rubin, Brian P ; Ellis, Ian O ; Chandarlapaty, Sarat ; Jungbluth, Achim A ; Brogi, Edi ; Weigelt, Britta ; Reis-Filho, Jorge S ; Rakha, Emad A

Abstract: AIMS Breast adenomyoepitheliomas (AMEs) are uncommon tumors. Most estrogen receptor (ER)-positive AMEs have mutations in PI3K pathway genes, whereas ER-negative AMEs usually harbor concurrent mutations affecting the HRAS Q61 hotspot and PI3K pathway genes. Here, we sought to determine the sensitivity and specificity of RAS Q61R immunohistochemical (IHC) analysis for detection of HRAS Q61R mutations in AMEs. METHODS AND RESULTS 26 AME (14 ER-positive, 12 ER-negative) previously subjected to massively parallel sequencing (n=21) or Sanger sequencing (n=5) of the HRAS Q61 hotspot locus were included in this study. All AMEs were subjected to IHC using a monoclonal (SP174) RAS Q61R-specific antibody, in addition to detailed histopathologic analysis. Nine ER-negative AMEs harbored HRAS mutations, including Q61R (n=7) and Q61K (n=2) mutations. 5/7 (71%) AMEs with HRAS Q61R mutations were positive by IHC, whereas none of the AMEs lacking HRAS Q61R mutations (n=17) were immunoreactive. RAS Q61R immunoreactivity was restricted to the myoepithelium in 80% (4/5) of cases, whereas one case displayed immunoreactivity in both the epithelial and myoepithelial components. RAS Q61R IHC-positive AMEs were associated with infiltrative borders ($P<0.001$), necrosis ($P<0.01$) and mitotic index in the epithelial ($P<0.05$) and myoepithelial ($P<0.01$) components. RAS Q61R IHC assessment did not detect Q61K mutations (0/2). CONCLUSIONS IHC analysis of RAS Q61R displays a high specificity (100%) and moderate sensitivity (71%) for detection of HRAS Q61R mutations in breast AMEs, and appears not to detect HRAS Q61K mutations. IHC analysis of RAS Q61R may constitute a useful marker in the diagnostic workup of ER-negative AMEs.

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MS FRESIA PAREJA (Orcid ID : 0000-0003-3748-8049)

DR FELIPE CORREA GEYER (Orcid ID : 0000-0002-9978-6650)

DR ZSUZSANNA VARGA (Orcid ID : 0000-0002-2855-983X)

PROFESSOR JORGE SERGIO REIS-FILHO (Orcid ID : 0000-0003-2969-3173)

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Immunohistochemical Assessment of *HRAS* Q61R Mutations in Breast Adenomyoepitheliomas

Fresia Pareja[1], Michael S. Toss[2], Felipe C Geyer[1], Edaise M. da Silva[1], Mahsa Vahdatinia[1], Ana Paula Martins Sebastiao[1], Pier Selenica[1], Austin Szatrowski[1], Marcia Edelweiss[1], Hannah Y. Wen[1], Raluca Mihai[2], Zsuzsanna Varga[3], Maria P. Foschini[4], Brian P Rubin[5], Ian O. Ellis[2], Sarat Chandarlapaty[6], Achim A. Jungbluth[1], Edi Brogi[1], Britta Weigelt[1], Jorge S. Reis-Filho[1], Emad A. Rakha[2]

[1]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA.
[2]Department of Histopathology, Nottingham University Hospitals NHS Trust, Nottingham, UK
[3]Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland.
[4]Department of Biomedical and Neuromotor Sciences, University of Bologna, Section of Anatomic Pathology, Bellaria Hospital, Bologna, Italy
[5]Department of Pathology, Cleveland Clinic, Cleveland, OH, USA
[6]Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA.

Correspondence to:

Dr. Jorge S. Reis-Filho, MD PhD FRCPath, Department of Pathology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA. Phone: +1-212-639-8054

Dr. Emad A. Rakha, MD, Department of Histopathology, Nottingham University Hospitals NHS Trust, Nottingham NG5 1 PB, UK. Phone: +44-0115 9691169

CONFLICTS OF INTEREST

JSR-F reports receiving personal/consultancy fees from Goldman Sachs and REPARE Therapeutics, membership of the scientific advisory boards of VolitionRx and Page.AI, and ad hoc membership of the scientific advisory boards of Roche Tissue Diagnostics, Ventana Medical Systems, Novartis, Genentech and InVicro, outside the scope of this study. All other authors declare no conflicts of interest.

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ABSTRACT

Aims: Breast adenomyoepitheliomas (AMEs) are uncommon tumors. Most estrogen receptor (ER)-positive AMEs have mutations in PI3K pathway genes, whereas ER-negative AMEs usually harbor concurrent mutations affecting the *HRAS* Q61 hotspot and PI3K pathway genes. Here, we sought to determine the sensitivity and specificity of RAS Q61R immunohistochemical (IHC) analysis for detection of *HRAS* Q61R mutations in AMEs.

Methods and results: 26 AME (14 ER-positive, 12 ER-negative) previously subjected to massively parallel sequencing (n=21) or Sanger sequencing (n=5) of the *HRAS* Q61 hotspot locus were included in this study. All AMEs were subjected to IHC using a monoclonal (SP174) RAS Q61R-specific antibody, in addition to detailed histopathologic analysis. Nine ER-negative

AMEs harbored *HRAS* mutations, including Q61R (n=7) and Q61K (n=2) mutations. 5/7 (71%) AMEs with *HRAS* Q61R mutations were positive by IHC, whereas none of the AMEs lacking *HRAS* Q61R mutations (n=17) were immunoreactive. RAS Q61R immunoreactivity was restricted to the myoepithelium in 80% (4/5) of cases, whereas one case displayed immunoreactivity in both the epithelial and myoepithelial components. RAS Q61R IHC-positive AMEs were associated with infiltrative borders (P<0.001), necrosis (P<0.01) and mitotic index in the epithelial (P<0.05) and myoepithelial (P<0.01) components. RAS Q61R IHC assessment did not detect Q61K mutations (0/2).

Conclusions: IHC analysis of RAS Q61R displays a high specificity (100%) and moderate sensitivity (71%) for detection of *HRAS* Q61R mutations in breast AMEs, and appears not to detect *HRAS* Q61K mutations. IHC analysis of RAS Q61R may constitute a useful marker in the diagnostic workup of ER-negative AMEs.

KEYWORDS: Breast, adenomyoepithelioma, immunohistochemistry, HRAS

INTRODUCTION

Breast adenomyoepitheliomas (AMEs) are a heterogeneous group of lesions with dual epithelial and myoepithelial cell differentiation, classically composed of glandular epithelial structures surrounded by myoepithelial cell proliferation[1-3]. Whilst most lesions are benign, a spectrum of lesions pertaining to this category has been described, ranging from purely benign to frankly malignant[2, 4-6]. Moreover, a subset of AMEs display overlapping morphology and immunophenotype with other lesions. At one end of the spectrum AMEs overlap with intraductal papillomas with myoepithelial cell hyperplasia, whereas, in the other end of the spectrum, malignant AME can mimic metaplastic breast carcinoma or what is so called malignant myoepithelial carcinoma[7-10], rendering a definite diagnosis of AME difficult to formulate at times[11]. The World Health Organization (WHO) guidelines describe malignant forms of AME and some cases were reported in the literature[5, 8], but clear criteria to distinguish this entity from the spindle cell metaplastic carcinoma apart from identification of benign AME component in the tumor remain to be defined[1, 2]. It is also our observation (EAR) that in a proportion of cases the concordance between morphology and immunoprofile of myoepithelial and epithelial cell components is low, making an accurate diagnosis and the distinction of each component a challenging task in such cases. The latter feature is important in cases showing atypia as the criteria for defining atypia are different between epithelial and myoepithelial cell components. Lack of definite diagnostic and molecular features of AME with their ambiguous nature and histogenesis leads to challenges in patients' management and outcome prediction.

The repertoire of genetic alterations affecting breast cancers has now been well-characterized, and includes recurrent mutations affecting *TP53, PIK3CA, PTEN* and *GATA3*[12-14]. In the large pool of breast carcinomas, and other rare tumors that originate in the breast, not uncommonly carry highly recurrent somatic genes alterations[15, 16]. We have recently shown that AMEs are underpinned by characteristic genetic alterations, which vary according to their estrogen receptor (ER) status[17]. ER-positive AMEs are associated with mutually exclusive *PIK3CA* or *AKT1* hotspot mutations, whereas up to 60% of ER-negative AMEs harbor concurrent *HRAS* Q61 hotspot mutations and mutations affecting either *PIK3CA* or *PIK3R1*[17]. Based on the results of this study[17] and a comparison with the repertoire of somatic mutations in common forms of breast cancer by The Cancer Genome Atlas[12] and The International Cancer Genome Consortium[14], we concluded that *HRAS* Q61 hotspot mutations are vanishingly rare in common forms of breast cancers, and

their presence in conjunction with PI3K-AKT pathway activation likely constitutes the driver genetic events in the development of AME.

There is an increasing interest in the application of immunohistochemistry (IHC) for the detection of specific hotspot mutations, particularly those that could be targetable, such as for instance *BRAF* V600E mutations[18] in melanoma[19], colorectal carcinoma[20] and papillary thyroid carcinoma[21], among others.

In this study we sought to determine the sensitivity and specificity of RAS Q61R immunohistochemical analysis for the detection of previously confirmed *HRAS* Q61R mutations in a series of AMEs. We also investigated whether specific histologic differences between RAS Q61R IHC-positive and –negative AMEs can be identified.

MATERIALS AND METHODS

Cases and DNA sequencing data

In this study, we included 26 breast AMEs with available material from the work by Geyer et al[17]. Representative histologic formalin-fixed paraffin-embedded (FFPE) blocks of breast AMEs included in this study were retrieved from the author's institutions. Approvals by the IRB and the local research committees have been obtained, and patient consent was obtained in accordance to the approved protocols. All cases were centrally reviewed by five pathologists with expertise in breast pathology (FCG, ME, IOE, and EAR and JSRF) for diagnosis confirmation following the WHO criteria[1]. Assessment of various histologic characteristics was conducted by three pathologists (FP, FCG and APMS), including growth pattern (tubular or papillary), tumor border (encapsulated, multinodular or infiltrative), epithelial and myoepithelial nuclear grade, which was evaluated following the Nottingham grading system of breast cancer[22], epithelial and myoepithelial mitotic rate, defined as the number of mitotic figures per mm^2, and presence or absence of necrosis. Whole-exome sequencing (n=9), MSK Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT)[23] targeted sequencing (n=12) and Sanger sequencing (n=5) data for the assessment of the mutational status of *HRAS* Q61 hotspot locus were retrieved from Geyer et al[17]. Immunohistochemical staining for p63 and ER were conducted in the study by Geyer et al[24]. ER status was assessed by IHC following the American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guidelines[25], using 1% of positive tumor nuclei as as cutoff for ER positivity. Hotspot mutations were annotated as per Chang et al[26].

Sanger sequencing

Areas with overgrowth of epithelial or myoepithelial cells of three AMEs were selected based on p63 expression and morphology. The epithelium and myoepithelium of only the selected areas was separately microdissected from eight micron-thick FFPE histological sections under a stereomicroscope (Olympus SZ61), following careful histologic review and use of the corresponding p63 IHC stains to highlight the myoepithelium, as reference. DNA was extracted using the DNAeasy Blood and Tissue Kit (Qiagen), following manufacturers' instructions. The presence of mutations affecting the *HRAS* Q61 hotspot locus was assessed by Sanger sequencing. In brief, PCR amplification was conducted using AmpliTaq Gold 360 Master Mix Kit (Life Technologies), as previously described[16]. Following purification with exoSAP-IT, PCR products were subjected to Sanger sequencing using previously validated primers[17] encompassing the *HRAS* Q61 hotspot locus (**Supplementary Table 1**). Sequence electropherograms corresponding to the forward and reverse strands were manually analyzed.

Immunohistochemistry

Immunohistochemical analysis was conducted using the monoclonal antibody SP174 (ab227658; Abcam, Cambridge, MA), an antibody generated against Q61R mutant NRAS, that also recognizes Q61R mutant HRAS and KRAS[27] due to the homology of the various RAS proteins[28]. The analyses were performed on a Leica Bond-3 automated stainer platform (Leica, Buffalo Grove, IL). In brief, 4-µm-thick deparaffinized FFPE tissue sections were incubated with primary antibody at 1:100 preceded by a heat-based antigen retrieval step employing a high pH buffer solution (ER2, Leica). As a secondary reagent, a polymeric kit (Refine, Leica) was employed. Appropriate positive and negative controls were included in each run. The evaluation of the IHC expression of mutant RAS Q61R was performed by three pathologists (FP, FCG and APMS) blinded to the results of the sequencing analysis.

Statistical analysis

Statistical analyses were performed using R v3.1.2. Fisher's exact test was used for comparisons between categorical variables. All tests were two-sided and *P*-values<0.05 were considered statistically significant.

RESULTS

Our study included 26 AMEs previously reported in our study by Geyer et al[17], of which 12 were ER-negative (**Table 1** and **Supplementary Table 2**). Nine and 12 AMEs have been previously

subjected to WES or MSK-IMPACT sequencing, respectively.[17] In addition, the presence of mutations affecting the *HRAS* Q61 hotspot locus had been previously interrogated using Sanger sequencing in five AMEs (**Supplementary Table 2**).[17] Seventy-five percent (9/12) of the ER-negative AMEs harbored *HRAS* Q61 hotspot mutations, including Q61R (n=7) and Q61K (n=2) mutations (**Fig. 1**). None of the ER-positive AMEs (n=14) were found to harbor *HRAS* Q61 hotspot mutations (**Fig 1**).

We sought to determine whether *HRAS* Q61R mutations could be detected by IHC. We subjected all 26 cases to immunohistochemical analysis with an antibody that detects Q61R mutant RAS (i.e. mutant NRAS, KRAS or HRAS)[27]. Seventy-one percent (5/7) of AMEs harboring *HRAS* Q61R mutations were immunoreactive using these antibodies, showing diffuse cytoplasmic and/or membranous staining, as previously reported in different tumor types[29, 30], and consistent with the reported localization of Ras isoforms in the plasma membranes and cytoplasmic organelles[31-33] (**Fig. 1** and **Fig. 2A-2D**). Notably, the two AMEs harboring *HRAS* Q61K mutations were negative by IHC (**Fig.1**). None of the 17 *HRAS* wild-type AMEs included in this study displayed immunoreactivity by IHC (**Fig. 1** and **Fig. 2E-2F**). RAS Q61R immunoreactivity was found to be restricted to the myoepithelial component in four cases (**Fig. 2A-2B**) and present in both the epithelial and myoepithelial component in one case (AM52; **Fig 2C-2D**). Taken together, these findings show that the immunohistochemical detection of *HRAS* Q61R mutations in ER-negative breast AMEs is moderately sensitive (71%) and highly specific (100%), whereas in ER-positive AMEs, this antibody was of limited use as no cases were found to harbor *HRAS* Q61 hotspot mutations and/or RAS Q61R protein expression.

We next sought to determine whether *HRAS* Q61R hotspot mutations would be present in the epithelium and myoepithelium of AMEs, or if they would be restricted to either histologic component. We conducted Sanger sequencing analysis of the *HRAS* Q61 hotspot locus in the separately microdissected epithelial and myoepithelial components of cases with available material (AM32, AM48 and AM52). Our analysis revealed the presence of *HRAS* Q61R hotspot mutations in both, epithelial and myoepithelial components of all three adenomyoeptiheliomas assessed (**Figure 3A-3F**). These findings support the notion that both the epithelium and myoepithelium of adenomyoepitheliomas are neoplastic.

RAS Q61R IHC-positive AMEs, compared to IHC-negative cases, more frequently displayed infiltrative tumor borders (100% vs 9.5%; $P<0.001$; **Table 1** and **Fig. 4A**), association with necrosis ($P<0.01$; **Table 1** and **Fig. 4B**) and higher epithelial ($P<0.05$) and myoepithelial ($P<0.01$)

mitotic index (**Table 1** and **Fig. 4C-4D**). All of the RAS Q61R IHC-positive AMEs were ER-negative (**Table 1** and **Fig. 4E-4F**), whereas the majority (67%) of RAS Q61R IHC-negative AMEs were ER-positive (P<0.05; **Table 1**). We observed no association of immunoreactivity for RAS Q61R with growth pattern or epithelial or myoepithelial nuclear grade (P>0.05; **Table 1**).

DISCUSSION

Here we evaluated the sensitivity and specificity of IHC analysis for the detection of mutated RAS Q61R protein in a set of 26 previous sequenced AMEs[17], encompassing 14 ER-positive AMEs and 12 ER-negative AMEs; of the 12 ER-negative AMEs, nine harbored mutations affecting the *HRAS* Q61 hotspot locus. Our findings show that IHC analysis has a high specificity and moderate sensitivity for identifying *HRAS* Q61R-mutated AMEs among ER-negative cases. The 2 cases Q61K *HRAS*-mutated cases, however, were not detected by IHC in this series. IHC evaluation showed no immunoreactivity in any of the 14 ER-positive AMEs, which lacked *HRAS* mutations providing further support to the remarkable specificity of IHC evaluation for the detection of Q61R *HRAS* mutations. Interestingly, RAS Q61R immunoreactivity was observed to be restricted to the myoepithelial component in four cases, and present in both the epithelial and myoepithelial components in one case. Nonetheless, our Sanger sequencing analyses of separately microdissected epithelium and myoepithelium of three AMEs revealed the presence of *HRAS* Q61R mutations in both histologic components. These findings are in agreement with our previous observations showing that *HRAS* Q61R mutations identified in ER-negative AMEs were clonal.[17] Hence, it is possible that the epithelial component of a subset of cases expressed a mutant *HRAS* Q61R, but at levels not detectable by immunohistochemistry. The molecular mechanisms underpinning the differences in expression and/ or immunohistochemical detection of *HRAS* Q61R mutations in the epithelium and myoepithelium of AMEs warrant further study.

Breast AMEs are a specific group of tumors both within the large pool of breast lesions, and within the wide spectrum of myoepithelial lesions[34]. Though not all AMEs show *HRAS* mutations and most of them are either negative or weakly positive for ER receptors[10], a strong correlation between *HRAS* mutation and the ER-negative subgroup has been previously reported by our group[17]. In our study, out of 12 ER-negative AMEs, 9 harbored *HRAS* mutations affecting the Q61 hotspot locus, whereas the remaining 3 were wild-type for *HRAS*. We have also recently observed, that a subset of AMEs lacking *HRAS* mutations, might be underpinned by *HMGA2* rearrangements, suggesting that a subset of AMEs could be related to salivary gland pleomorphic adenomas[35]. The relevance of the genetic alterations underpinning AMEs is of practical importance when faced with differential diagnosis dilemmas, such as the discrimination of AMEs

from other ER-negative breast tumors, including metaplastic carcinomas, adenoid cystic carcinomas and pleomorphic adenomas, with which adenomyoepitheliomas may show morphologic overlap[36]. The detection of *HRAS* Q61R mutations in AMEs with ambiguous morphology may help settle such challenging cases. Nonetheless, given that RAS Q61R immunohistochemical assessment has a moderate sensitivity for the detection of *HRAS* Q61R mutations and does not detect *HRAS* Q61K mutations, along with the fact that a subset of approximately 30%-40% of ER-negative AMEs lack *HRAS* mutations[17], the diagnosis of AME should not be ruled out in cases displaying the typical histologic features but lacking RAS Q61R expression by by immunohistochemistry.

Though it is generally considered that triple-negative breast neoplasms are biologically more aggressive, this is not the case across the entire spectrum of these tumors[37]. AMEs are considered to be among the least aggressive breast neoplasms and are generally cured by local excision alone[1]. A spectrum of these lesions appears to exist with some AMEs showing unpredictable behavior. Nodal metastasis has been reported in AMEs ranging from benign to atypical cases with no frank malignant histological features[4, 5, 14, 38]. There are no established criteria on how to catalogue these lesions[1], though general features such as mitotic activity, necrosis, cellular pleomorphism, peripheral invasive border and overgrowth of myoepithelium are suggested[2]. In our study, out of 7 cases categorized as having an infiltrative border, five AMEs were immunoreactive for mutant RAS Q61R. A sixth case with infiltrative borders showed the Q61K mutation, making for a total of 6 out of 7 cases with infiltrative border associated with *HRAS* mutations. The presence of necrosis was also recorded in all five cases with IHC positivity for RAS Q61R, and compared to IHC-negative AMEs, RAS Q61R IHC-positive cases had a higher mitotic index in both the epithelial and myoepithelial components. These findings corroborate the more aggressive histologic features associated with lack of ER expression and the presence of *HRAS* Q61 hotspot mutations in AMEs[17].

Limitations of the current study include the relatively low number of AMEs harboring *HRAS* mutations and the lack of follow-up information, due to the multi-institutional nature of our cohort. Despite these limitations, our study demonstrates that the IHC analysis of HRAS Q61R displays a high specificity and moderate sensitivity for the detection of *HRAS* Q61R mutations in breast AMEs, and it appears not to detect *HRAS* Q61K mutations. Having previously concluded that these mutations likely constitute founder genetic events in the development of ER-negative AMEs[17], detection of immunoreactivity for RAS Q61R mutations would aid in distinguishing these unusual breast lesions. Given the fact that in the context of primary breast tumors, *HRAS* Q61R

mutations appear to be restricted to ER-negative AMEs, the immunohistochemical assessment of HRAS Q61R may represent a useful marker in the diagnostic workup of these lesions.

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LIST OF ONLINE SUPPORTING INFORMATION

Supplementary Table 1. Primers used for Sanger Sequencing analysis of the *HRAS* Q61 hotspot locus.

Supplementary Table 2. Sequencing platform, *HRAS* mutational status, and clinicopathologic characteristics of the 26 breast adenomyoepitheliomas included in this study.

AUTHORS' CONTRIBUTIONS

JSR-F and EAR conceived the study. ZV, MPF, BPR, IOE, EB AND EAR contributed with cases. FP, FCG, APMS, ME, IOE, JRSF and EAR reviewed the cases. FP, FCG, EMdS, MV, APMS, PS, AS, HYW, RM and SC analyzed and interpreted the data. FP, MT and EAR wrote the first manuscript, which was reviewed by all coauthors.

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Table 1. Clinicopathologic features of the breast adenomyoepitheliomas studied according to RAS Q61R immunoreactivity

Clinicopathologic feature		RAS Q61R IHC		*P* value*
		Positive (*n*=5)	**Negative (*n*=21)**	
ER	Positive	0.0% (0)	66.7% (14)	**0.012**
	Negative	100.0% (5)	33.3% (7)	
Growth pattern	Tubular	60.0% (3)	76.2% (16)	0.5875
	Papillary	40.0% (2)	23.8% (5)	
Tumor border	Encapsulated	0.0% (0)	23.8% (5)	**0.0003**
	Multinodular	0.0% (0)	66.7% (14)	
	Infiltrative	100.0% (5)	9.5% (2)	
Nuclear grade (epithelium)	Low	0.0% (0)	23.8% (5)	0.0816
	Intermediate	0.0% (0)	38.1% (8)	
	High	100.0% (5)	38.1% (8)	
Nuclear grade (myoepithelium)	Low	0.0% (0)	4.8% (1)	0.467
	Intermediate	20.0% (1)	47.6% (10)	
	High	80.0% (4)	47.6% (10)	
Epithelial mitoses/ mm^2	<=0.8	40.0% (2)	81% (17)	**0.0494**
	>0.8 but <=2.1	40.0% (2)	19% (4)	
	>2.1	20.0% (1)	0% (0)	
Myoepithelial mitoses/ mm^2	<=0.8	0.0% (0)	85.7% (18)	**0.0013**
	>0.8 but <=2.1	60.0% (3)	4.8% (1)	
	>2.1	40.0% (2)	9.5% (2)	
Necrosis	Absent	0.0% (0)	76.2% (16)	**0.0038**
	Present	100.0% (5)	23.8% (5)	

*Two-tailed Fisher's exact test

FIGURE LEGENDS

Figure 1. Estrogen receptor-negative breast adenomyoepitheliomas harbor recurrent *HRAS* Q61R hotspot mutations, which are detectable by immunohistochemistry, co-occurring with mutation in PI3K genes.

Heatmap depicting somatic mutations affecting the *HRAS* Q61 hotspot locus and immunoreactivity for RAS Q61R in the 26 breast adenomyoepitheliomas included in this study. Cases are shown in columns and genes in rows. Mutations are color-coded according to the legend. Estrogen receptor status and the sequencing platform used are depicted in phenotype bars (top).

Figure 2. Detection of *HRAS* Q61R hotspot mutations in breast adenomyoepitheliomas by immunohistochemistry.

(**A-B**) Representative micrographs of (**A**) hematoxylin and eosin-stained (H&E) sections and (**B**) RAS Q61R immunohistochemical expression of AM8 showing immunoreactivity in the myoepithelial component. (**C-D**) Representative micrographs of (**C**) H&E sections and (**D**) RAS Q61R immunohistochemical expression of AM52 displaying immunoreactivity in both the epithelial and myoepithelial component. (**E-F**) Representative micrographs of (**E**) H&E stained sections and (**F**) lack of RAS Q61R immunohistochemical expression in AM3. Scale bars, 50 μm.

Figure 3. *HRAS* Q61R mutations are present in the epithelial and myoepithelial components of breast adenomyoepitheliomas.

(**A-F**) Representative micrographs of hematoxylin and eosin-stained (H&E) sections (left), p63 (center) and RAS Q61R (right) immunohistochemical expression in the epithelial and myoepithelial components of adenomyoepitheliomas AM32 (**A-B**), AM48 (**C-D**) and AM52 (**E-F**). Representative Sanger electropherograms of the *HRAS* Q61 hotspot locus of separately microdissected epithelial and myoepithelial components of AM32 (epithelium, **A**; myoepithelium, **B**), AM48 (epithelium, **C**; myoepithelium, **D**), and AM52 (epithelium, **E**; myoepithelium, **F**).

Figure 4. Histopathologic characteristics of breast adenomyoepitheliomas immunoreactive for RAS Q61R.

Representative micrographs of (**A**) hematoxylin and eosin-stained (H&E) sections of AM9 showing infiltrative borders, (**B**) AM52 displaying areas of necrosis, (**C**) high epithelial and myoepithelial nuclear grade and (**D**) frequent epithelial and myoepithelial mitotic figures. (**E-F**) Representative micrographs of (**E**) H&E stained sections and corresponding (**F**) estrogen receptor (ER) immunostain of AM8. Scale bars, (A) 200 μm, (B) 100 μm, (C-D) 20 μm and (E-F) 50 μm.

Figure 1

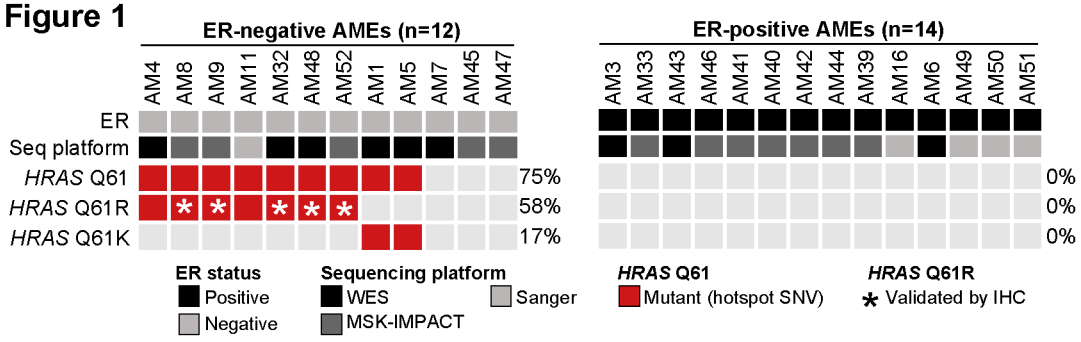

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Figure 2

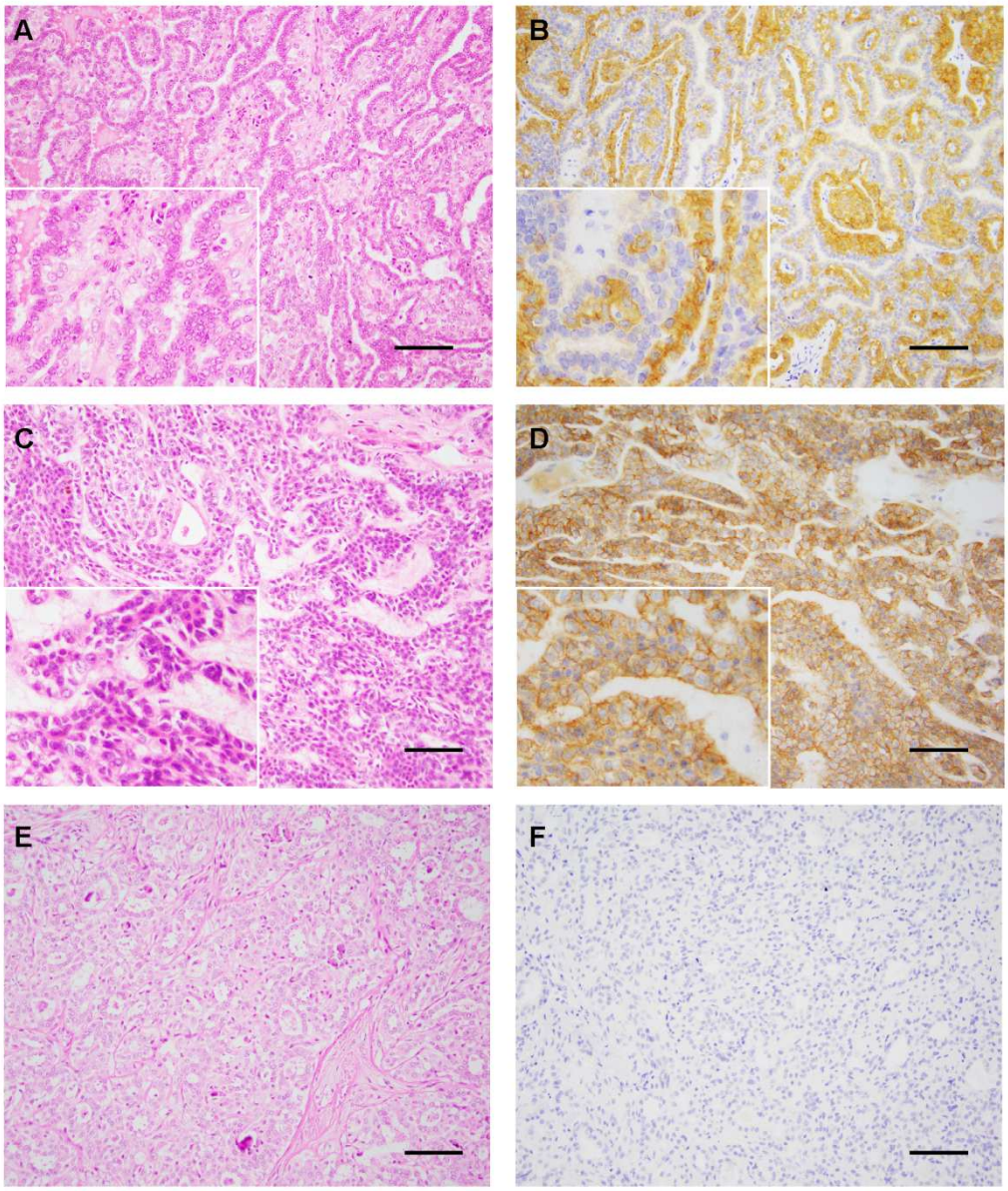

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Figure 3

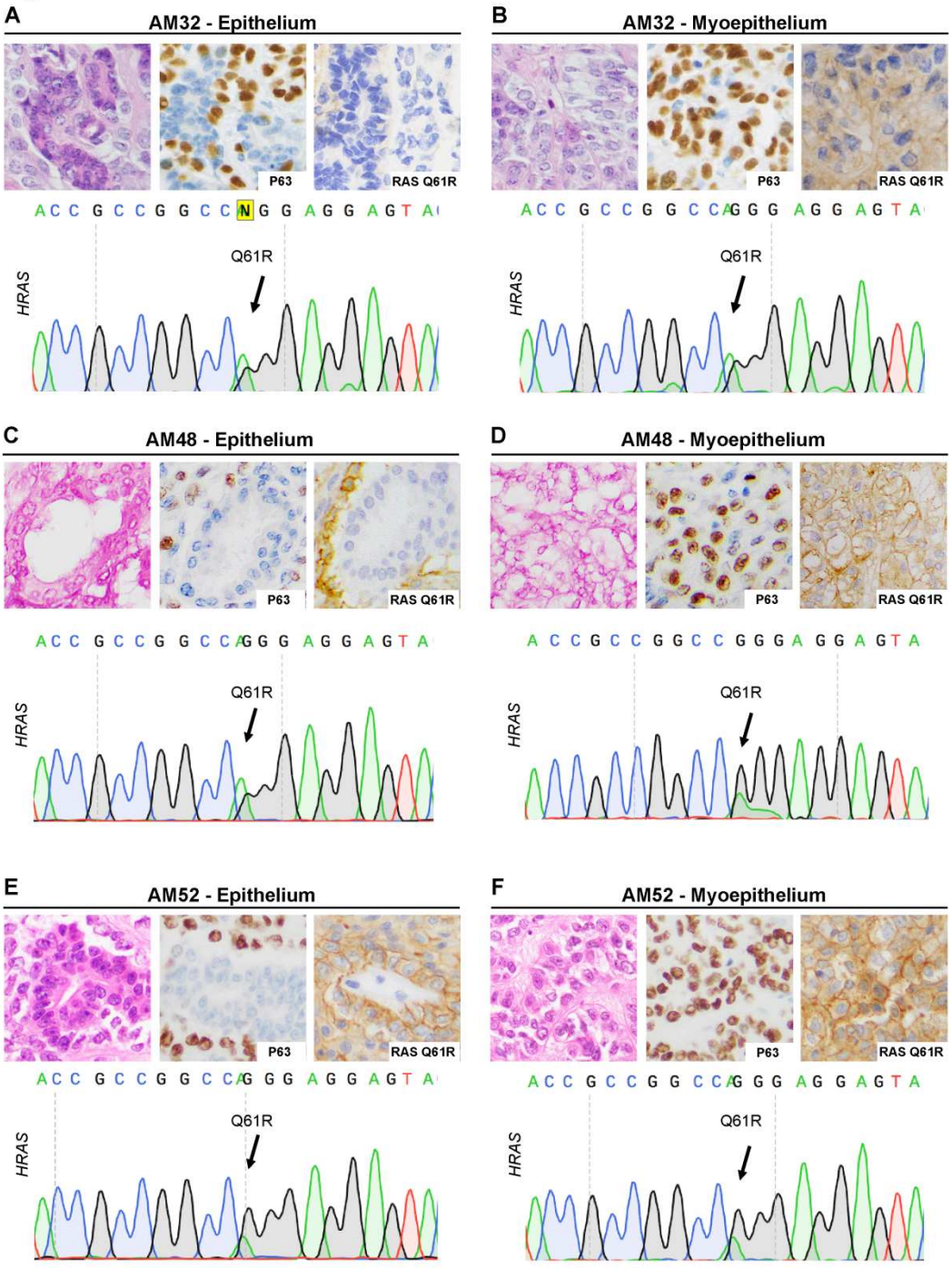

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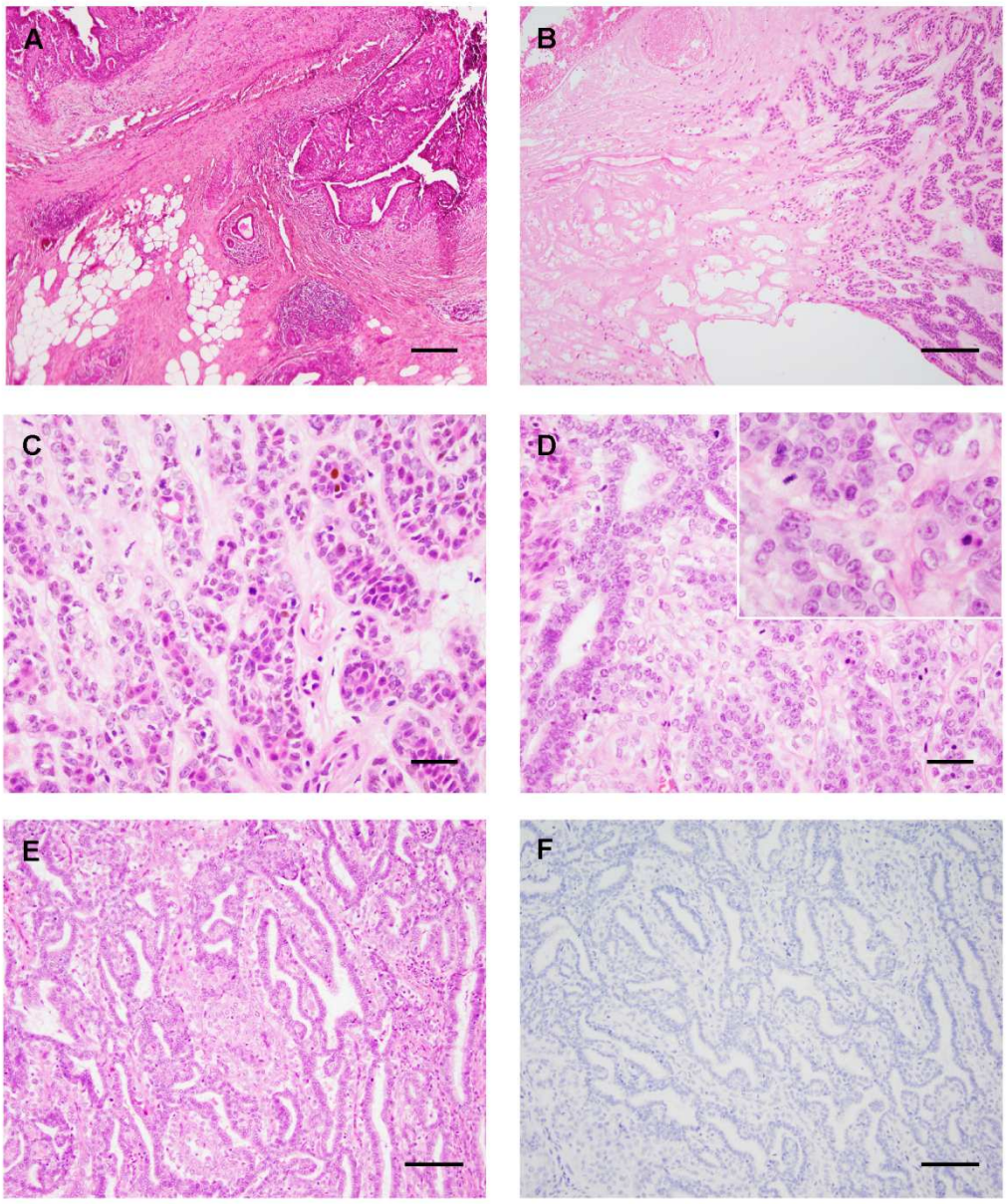
Figure 4



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